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Molecular Alterations Underlying the Enhanced Disruption of Spermatogenesis by 2,5-Hexanedione and Carbendazim Co-exposure

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Abstract

The current study investigated the co-exposure effects of 2,5-hexanedione (HD) and carbendazim (CBZ) on gene expression underlying the enhanced pathology previously observed. Adult male rats were exposed to HD (0.33 or 1%) followed by CBZ (67 or 200 mg/kg), and testis samples were collected after and 24 h. Microarray analysis at 3 h revealed that CBZ and HD interact in an agonistic, or synergistic, way at the gene level. Further analysis of candidate genes by qRT-PCR at both 3 and 24 h after co-exposure, revealed that *Lox11* and *Clca2/Clca4l* were both decreased in expression. Immunohistochemical analysis of *Lox11* at 24 h revealed that *Lox11* is localized to the seminiferous tubules, with the most intense staining in the basement membrane, blood vessels, and acrosomes, with the relative intensity reflecting the gene level changes at 3 h. These findings provide candidate genes for further investigation of the testicular response to damage.

Keywords

testis; carbendazim; 2,5-hexanedione; microarray; co-exposure; *Lox11*; *Clca2/Clca4l*

1. Introduction

The cellular and molecular targets and dose level of each toxicant within a chemical mixture all play a role in determining the biological responses following exposure. There is an emerging need for improved methods for risk assessment and a better understanding of toxicological consequences of mixed exposures, given that most real world exposures involve more than one chemical. Recent work has begun to elucidate how critical the dose

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and cellular and subcellular targets are during co-exposure to testicular toxicants. This work utilized an established co-exposure paradigm, which involves an 18 day priming exposure of adult male rats to the Sertoli cell toxicant 2,5-hexanedione (HD) followed by acute exposure to either a direct-acting germ cell toxicant or a second Sertoli cell toxicant. Utilizing this exposure paradigm to study co-exposure responses, it was determined that co-exposure to the Sertoli cell toxicant (HD) and the germ cell toxicant x-radiation (x-ray) results in an attenuation of germ cell toxicity when compared with x-ray exposure alone [1]. In this co-exposure scenario we hypothesize that HD induces an adaptive response of the seminiferous epithelium, which helps render the germ cells resistant to x-ray-induced damage. In contrast to this co-exposure response, co-exposure to two Sertoli cell toxicants, HD and carbendazim (CBZ), results in synergistic effects on testicular injury, much greater than the single toxicant exposures, manifested as enhanced seminiferous tubule diameter, vacuolization, sloughing, and germ cell apoptosis [2].

While HD and CBZ share the same target, Sertoli cell microtubules, these two toxicants have opposing effects on microtubules. HD, a metabolite of the commonly used solvents *n*-hexane and methyl *n*-butyl ketone (2-hexanone), causes Sertoli cell dysfunction by promoting rapid assembly and enhanced stability of microtubules [3]. Consequences of HD exposure include spermatid head retention, Sertoli cell vacuolization, and decreased seminiferous tubule fluid, ultimately resulting in germ cell loss and sloughing [3, 4]. HD-induced testicular toxicity requires at least 2 weeks of exposure before the manifestation of pathology [3], which differs from the more rapid onset of pathology following CBZ exposure. A single high dose of CBZ results in testicular alterations within a few hours, including increased testis weights, increased seminiferous tubule diameter, and sloughing of the seminiferous epithelium [2, 4]. CBZ is the toxic metabolite of the benzimidazole fungicide benomyl, which elicits testicular toxicity by inhibiting, rather than promoting microtubule polymerization [5, 6].

Mechanistic investigations into the molecular changes underlying the phenotypic consequences of HD and x-ray co-exposure led to the establishment of a methodology for the analysis and interpretation of microarray data from animals co-exposed to multiple dose levels of each toxicant [7]. This same approach was applied in the current study to investigate the dose and co-exposure effects on gene expression following combined HD and CBZ exposure and to determine the molecular mechanisms underlying the pathologic changes. It was hypothesized that HD and CBZ co-exposure would result in synergistic or agonistic effects at the molecular level, reflective of the agonistic phenotypic effects of this co-exposure. The log₂-expression values obtained by microarray analysis performed with testis tissue of co-exposed adult male rats were analyzed using LIMMA and summarized across all treatment groups to determine the effect of HD in excess of CBZ. These summarized linear contrasts were examined to identify individual genes and biological pathways where HD modification of CBZ-induced gene alterations was the greatest. Several genes of interest were further analyzed by qRT-PCR and immunohistochemistry to begin to better understand and mechanistically explain the synergistic toxicity elicited by HD and CBZ co-exposure. This is an important area of investigation, which has provided valuable information regarding the molecular profiles of toxicants with synergistic phenotypic effects as compared to the molecular profiles of toxicants with antagonistic phenotypic effects. These are significant pieces of information in the field of mixtures research.

2. Materials and Methods

2.1 Animals

Adult male Fischer 344 rats weighing 200–250g were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, rats were acclimated for 1 week prior to use

and maintained in a temperature and humidity controlled environment with a 12 h alternating dark-light cycle. All rats were housed in community cages with free access to water and Purina Rodent Chow 5001 (Farmer's Exchange, Framingham, MA). The Brown University Institutional Animal Care and Use Committee approved all experimental animal protocols in compliance with National Institute of Health guidelines.

2.2 Toxicant Exposure

HD was administered in drinking water ad libitum for 18 days at concentrations of 0.33% and 1% using a previously established treatment protocol [2]. On day 17, animals (n=4 for each treatment group) were administered CBZ by gavage, at a dose of 67 mg/kg or 200 mg/kg in corn oil at a dose volume of 2 ml/kg. At either 3 h or 24 h after treatment with CBZ, following continued HD drinking water exposure, rats were euthanized by CO₂ asphyxiation and half of the right testis was homogenized in Tri Reagent (Sigma-Aldrich, St. Louis, MO), snap frozen in liquid nitrogen, and stored at -80°C. The remaining testis tissue was fixed in neutral buffered formalin for histological examination.

2.3 RNA Isolation and Microarray Hybridization

Using tissues collected at 3 h after treatment with CBZ, RNA was isolated from testes homogenized in Tri Reagent using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Complementary (cDNA) was synthesized from 2.5µg total RNA and purified using the Affymetrix One-Cycle Target Labeling and control reagents kit (Affymetrix, Santa Clara, CA) according to manufacturer's protocol. Equal amounts of purified cDNA per sample were used as the template for subsequent in vitro transcription reactions for complementary RNA (cRNA) amplification and biotin labeling using the Affymetrix GeneChip IVT labeling kit (Affymetrix) included in the One-Cycle Target Labeling kit (Affymetrix). cRNA was purified and fragmented according to the protocol provided with the GeneChip Sample Cleanup module (Affymetrix). All GeneChip arrays (Rat Genome 230 2.0 arrays) were hybridized, washed, stained, and scanned using the Complete GeneChip Instrument System according to the Affymetrix Technical Manual.

2.4 Microarray Data Analysis

Affymetrix CEL files were pre-processed by GCRMA background correction [8], quantile normalization and Robust Microarray summarization, resulting in a single log₂-transformed expression measure for each of 31,099 genes. The expression measures were analyzed as previously described [7] to facilitate the detection of nonlinear effects of exposure and interactions of co-exposure on mRNA expression. This method of analysis resulted in the generation of a summary statistic with the interpretation of an estimated aggregate HD effect in excess of CBZ, e.g. up-regulation (positive) or down-regulation (negative) by HD. The overall linear trend in CBZ was also summarized by fitting the equivalent saturated model reparameterized using polynomials in exposure dose (i.e. linear and quadratic terms for each exposure together with their interactions), and extracting the linear CBZ term. To control for multiple comparisons, Q-values representing false discovery rates (FDR) were computed from the collection of all 31,099 P values using the *qvalue* package in R [9].

2.5 Quantitative Real Time- Polymerase Chain Reaction (qRT-PCR) Analysis

Using tissues collected at both 3 h and 24 h after treatment with CBZ, Stat-60 reagent (Tel-Test, Friendswood, TX) was used to extract total RNA from whole testis tissue according to the manufacturer's protocol. RNA concentrations were determined using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. cDNA was synthesized from total RNA

isolated from each sample using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. For the detection of MACRO domain containing 1 (Macro1), dipeptidylpeptidase 7 (Dpp7), SH3 and multiple ankyrin repeat domains 3 (Shank3), chloride channel calcium activated 2/chloride channel calcium activated 4-like (Clca2/Clca4l), lysyl oxidase-like 1 (Loxl1), and tubulin beta 3 (Tubb3) the cDNA templates were amplified using QuantiTect® Primer Assays (Qiagen, Valencia, CA). These primers are pre-optimized and bioinformatically validated. Each sample was run in triplicate in 25 µl reactions. Relative mRNA levels of each target gene were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Log₂-transformed relative expression ratios were calculated using the ddCt method.

2.6 Immunofluorescent Staining and Densitometric Analysis

Rat testes (control, 1% HD, 200 mg/kg CBZ, and 1% HD + 200 mg/kg CBZ; n=6) were collected 24 h after treatment and cryopreserved in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA). Sections (8µm) were fixed in acetone for 10 minutes, air dried for 5 minutes and then washed in phosphate buffered saline (PBS). Endogenous activity was blocked with 6% goat serum for one hour and then incubated overnight at 4°C with rabbit anti-LOXL1 primary antibody (0.5µg/mL) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Alexa Fluor 568 goat anti-rabbit (2.5ug/mL) (Invitrogen, Carlsbad, CA) was applied to detect the anti-LOXL1 antibody. Sections were counterstained and coverslipped with Vectashield® Hard Set mounting medium with DAPI (Vector Laboratories Inc., CA, USA). Images of Stage IV seminiferous tubules were captured on an Axio Imager.M1 microscope, with an AxioCam MRm camera and Axio Vision 4.8 Software, (Carl Zeiss, Inc, Germany) at 40× magnification and 60 millisecond (ms) exposure for densitometric quantification of the fluorescent staining. The 60 ms exposure time was determined following an exposure time course study that was performed to determine the linear range capabilities of the gray scale.

Images of the anti-LOXL1 staining in Stage IV seminiferous tubules were blinded and uploaded into Image J (NIH, Bethesda, MD) as black and white JPEGs for densitometric quantification of the fluorescent staining. Using the Image J software, a set of intersecting lines was drawn over each seminiferous tubule to separate each cross-section into quadrants. Within each quadrant, two circles (with standard areas) were drawn over the basement membrane and over the closest acrosome to the basement membrane. The mean gray value of the area of each circle was measured and recorded. The mean gray values for both the basement membrane and the acrosome from the four quadrants were averaged together. The averaged value for the acrosome was then divided by the averaged value for the basement membrane to create a ratio of mean gray value, which acts as a control to compensate for staining differences between sections. The ratios of mean gray value for each stage IV seminiferous tubule were then averaged, resulting in one ratio of mean gray value per animal.

2.7 Statistical analysis

qRT-PCR data were analyzed using a one-way analysis of variance (ANOVA) with Bonferonni post hoc analysis. The analyses were performed separately for each gene, comparing the expression data among all treatment groups (control, HD, CBZ, HD + CBZ) for each individual gene. Immunofluorescent quantification data was also analyzed by one-way ANOVA with Bonferonni post hoc analysis. *p* values <0.05 were considered significant.

3. Results

3.1 Estimates of HD Effects on Gene Expression

Initial studies investigating co-exposure to the two Sertoli cell toxicants HD and CBZ revealed that they interact to produce synergistic effects on testicular toxicity, at the phenotypic level. To determine if these toxicants similarly interact at the molecular level to affect how they each contribute to the gene expression profile during co-exposure, a summary statistic was used, as previously described [7], to estimate the extent to which HD modifies gene expression above and beyond the CBZ-induced gene expression for each gene during co-exposure. This approach facilitates the identification of genes that exhibit enhanced alteration (up- or down-regulation) by HD co-exposure at the molecular level that mirrors the enhanced toxicity with combined HD and CBZ co-exposure as compared to CBZ alone. CBZ linear effects (the summarized linear trend in CBZ) on gene expression were also determined to provide an indication of the expression induced by CBZ during co-exposure so that we can understand if the HD effect on top of CBZ is an attenuation or enhancement of these changes. The time point for microarray analysis was 3 h following CBZ treatment, because this represents a time prior to the manifestation of pathology due to CBZ exposure.

HD exerted a significant modification of CBZ-induced gene expression alterations ($FDR < 0.05$) for 5 genes: *Dpp7*, *Macrocl1*, *Shank3*, *Clca2/Clca4l* and *Loxl1* (Table 1 and Figure 1). All 5 of these genes were negatively affected by HD, which represented an enhanced down-regulation on top of CBZ, as indicated by the linear CBZ effects and agonistic interactions in Table 1. An agonistic vs antagonistic interaction is described by Campion et al [7]. Genes that are significantly affected by CBZ, defined as genes with $FDR < 0.05$ for linear CBZ effects, are listed in Table 2. There are 16 genes with significant CBZ linear effects, and of interest, *Clca2/Clca4l* is the only gene that is significantly affected by both HD and CBZ as detected by microarray analysis.

3.2 qRT-PCR of Candidate Genes

To confirm the co-exposure effects on gene expression detected by gene array analysis, qRT-PCR analysis was performed focusing on the 5 genes that were significantly altered by HD in excess of CBZ at the high dose levels. The expression of *Tubb3* was also investigated since tubulin is anticipated to be affected by HD and CBZ. At 3 h after CBZ exposure (Figure 2) there were similar trends in expression for all of the genes, with decreased expression occurring with combined CBZ and HD exposure. *Macrocl1* gene expression was significantly increased compared to control (1.52-fold change) after HD treatment alone, while expression was significantly decreased as compared to HD alone with co-exposure (0.9X control). Comparing this to the microarray results at the same dose levels, *Macrocl1* was reduced to 0.6X control with co-exposure, but with no change in expression in the other treatment groups. *Dpp7* was significantly reduced with co-exposure (0.8X control) as compared to HD and CBZ alone, similar to the decrease of 0.6X control detected on the array. Reductions in *Clca2* expression with co-exposure were 0.7X control (Figure 2) and 0.57X control (Figure 1) as detected by qRT-PCR and microarray, respectively. No significant changes in *Shank3*, *Loxl1* and *Tubb3* expression were detected at the 3 h time point, however large decreases with HD and CBZ combined exposure were observed for *Shank3* and *Loxl1* (~0.6X of control for both genes), which reflect the decreases in expression detected by microarray analysis.

This analysis was extended to a later time point, 24 h, to further investigate the expression of *Clca2/Clca4l*, *Loxl1* and *Shank3*, which all exhibited the greatest degree of alteration with co-exposure and were likely to play a role in the co-exposure response based on their known

functions. Focusing on the effect of CBZ and how this is modified by HD co-exposure, there were strong trends for enhanced CBZ effects on gene expression with the addition of HD treatment (Figure 3). HD exposure alone was not investigated at the 24 h time point (18 days of HD exposure) because this was expected to be very similar to the data obtained at the 3 h time point (17 days and 3 h of HD exposure). Both *Clca2* and *Loxl1* exhibited slight decreases with CBZ exposure alone and much greater decreases with co-exposure (0.35X control and 0.6 X control for *Clca2* and *Loxl1*, respectively). The 24 h results with Shank3 were the opposite of the 3 h findings (increase in expression with co-exposure at 24 h compared to a decrease at 3 h) and exhibited the lowest magnitude of gene alteration.

3.3 *Loxl1* Immunostaining

Immunohistochemistry was performed to evaluate the protein expression of *Loxl1* after CBZ and HD co-exposure. Although both *Clca2/Clca4l* and *Loxl1* exhibited large changes in gene expression after combined exposure, only *Loxl1* was further investigated at the protein level due to the absence of commercially available antibodies that recognize rat *Clca2/Clca4l*. Representative images of *Loxl1*-stained seminiferous tubules, at approximately stage IV, are shown in Figure 4. Acrosome staining was more easily identifiable in stage IV seminiferous tubules, compared to tubules of other stages; therefore, stage IV seminiferous tubules were the primary focus of the staining analysis. *Loxl1* localized to several areas in the seminiferous tubules in all exposure groups, including the acrosomes (where the most intense staining was observed), the tails of immature spermatozoa, the cytoplasm of Sertoli cells, the basement membrane of the seminiferous tubule, and blood vessels. This staining was more intense in the 1% HD (Figure 4B) and 200mg/kg CBZ (Figure 4C) exposed testis tissues, in comparison to the control (Figure 4A). The staining appeared to decrease overall in the combined 1% HD and 200mg/kg CBZ exposed testis as seen in Figure 4D.

To further explore the alterations in *Loxl1* protein levels in these tissues, a densitometric analysis method was developed to quantify the intensity of *Loxl1* staining. The quantification of *Loxl1* protein levels at 24 h following CBZ exposure, expressed as mean gray value, is shown in Figure 5. The 24 h time point was chosen for analysis of protein levels, because the 3 h gene expression time point would not result in immediate alterations of protein levels. There is an apparent trend in *Loxl1* protein expression, which increases with the individual exposures of 1% HD or 200mg/kg CBZ and appears to decrease with the combined exposure of 1% HD and 200mg/kg CBZ. Although this trend is not statistically significant, it mimics the gene expression data that was obtained for *Loxl1* at the 3 h time point.

4. Discussion

The cellular and subcellular targeting of the individual toxicants in a mixture determine the toxicological outcome. The current study has demonstrated that CBZ and HD interact in an agonistic way at both the phenotypic level and at the gene level. Combined exposure to HD and CBZ has synergistic effects on the expression of specific genes, including *Clca2/Clca4l* and *Loxl1*, whose altered expression may underlie the enhanced testicular toxicity. *Clca2/Clca4l* and *Loxl1* were identified as candidate genes involved in the enhanced co-exposure response through microarray analysis. Similar to a previous study [7], microarray data were summarized across different dose levels and treatment groups (toxicant combinations) using a statistical method that estimates the extent to which HD modifies gene expression above and beyond the CBZ-induced gene expression for each gene during co-exposure. This allowed for the identification of genes that are more altered in expression by co-exposure as compared to CBZ alone. These genes are likely to be related to the enhanced pathology. All 5 genes that were significantly altered by HD on top of CBZ exhibited agonistic, or synergistic, gene expression alterations with co-exposure as detected by gene array analysis,

which mirrors the synergistic effects of co-exposure on testicular toxicity. Although previous studies have demonstrated that microarray analysis using RNA isolated from whole testis tissue may not provide the most reliable information for hypothesis generation [10], both *Clca2* and *Loxl1* proved to be promising genes of interest likely to play a role in the enhanced co-exposure toxicity.

Although most of the microarray expression alterations were confirmed by qRT-PCR, there were some discrepancies. Some of these differences may be due to the differences in sensitivity and the different dynamic ranges of the two different technologies. It is also important to remember that the estimated “effects” used to identify candidate genes for additional analysis were derived from the microarray expression data for all 9 different treatment groups across different dose levels, while the qRT-PCR was performed only on the high doses. As described in the results section, the microarray fold-changes for those specific treatment groups are quite similar to the qRT-PCR-detected gene alterations in those same treatment groups. The only real discrepancy between the 3 hr qRT-PCR results and the microarray results (also obtained at the 3 hr time point) is the significant increase in *MacroD1* detected by qPCR after 1% HD exposure. No significant changes were detected by array for this treatment group. All other significant changes detected by qRT-PCR confirm the microarray results. The expression pattern of *Tubb3* (tubulin, beta 3), as measured by qRT-PCR, provides confidence in these data. Tubulin synthesis is controlled by autoregulation, whereby non-polymerized, free tubulin monomers provide feedback, regulating mRNA levels of tubulin [11]. Since CBZ and HD inhibit or promote microtubule assembly, respectively, one would expect CBZ to decrease tubulin mRNA levels and HD to increase tubulin mRNA levels. As would be expected, an increase in tubulin mRNA levels after HD exposure is observed in the current study, with only a slight decrease detected after CBZ exposure. These tubulin expression results provide confidence in the quality of the RNA used for microarray analysis and the resulting data.

Loxl1 catalyzes the oxidation of lysine residues of collagen fibrils and elastins in the extracellular matrix, which controls the cross linking and deposition of elastins. *Loxl1* is localized specifically to sites of elastogenesis and plays a role in elastin homeostasis [12]. Interestingly, *Loxl1* $-/-$ male mice exhibit lower sperm production and reduced fertility, with no apparent histologic differences [13]. *Loxl1* appears to play a role in male sexual development and fertility, but the specific mechanism(s) are currently unknown. The substantial reduction in *Loxl1* mRNA in the present study suggests that this gene product may also play a role in the toxicological response of the testis. A closely related family member to *Loxl1*, lysyl oxidase (LO), is able to prevent the activation of NF κ B by inhibiting the signaling pathways that lead to its activation [14], so one would anticipate that reduced LO or *Loxl1* levels would lead to greater activation of NF κ B. Given that NF κ B is pro-apoptotic in the testis [15], the reduction in *Loxl1* observed in the current study corresponds with, and may mechanistically explain, the enhanced injury and apoptosis that occurs following HD and CBZ co-exposure. One might also speculate that altered *Loxl1* expression during injury may impact germ cell support through alterations in cell-matrix interactions and cell junction dynamics related to elastic fiber homeostasis. Further mechanistic studies are required to better understand the role of *Loxl1* in the testis, both under normal conditions and following toxicant exposure.

In addition to the detected changes in *Loxl1* expression after co-exposure, altered expression of the chloride channel *Clca2/Clca4l* in the testis likely has significant consequences, although the specific role and localization of this particular chloride channel in the testis has not yet been investigated. Chloride channels have been demonstrated to play a role in regulating the volume of spermatozoa, the regulation of seminiferous tubule fluid formation, and in controlling the ionic environment in the testis [16, 17]. The survival of germ cells and

the proliferation of spermatogonia is dependent on the production of an acidic microenvironment. Lactate secretion by Sertoli cells helps to maintain this acidic microenvironment and the chloride currents in Sertoli cells may be involved in the proton-linked lactate production in Sertoli cells [18, 19]. Other members of the Clca protein family appear to play a role as cell-cell adhesion molecules, which may be important in the testis [20]. The critical role of chloride channels in the testis is observed in the severe degeneration in mouse testes that occurs when chloride channel expression is disrupted [19].

While Clca2/Clca4l is a strong candidate for further analysis, Clca2/Clca4l staining was not performed because there are no commercially available antibodies to detect the rat protein. The quantification of Loxl1 immunostaining reflects the gene expression as measured by qRT-PCR at 3 h, however the changes in protein levels were not large in magnitude or statistically significant. This non-significance may be attributed to the 24 h time point at which protein expression was examined. The greatest change in Clca2/Clca4l gene expression occurs at 24 h, with the magnitude of change for Loxl1 being consistent between 3 and 24 h. This may reflect a sustained alteration in Loxl1 expression over these time points, or may indicate a peak in gene expression alteration between these 2 time points. This may indicate that significant alterations in Loxl1 protein levels would follow at a later time point.

The localization of Loxl1 within the testis has not been previously investigated. Loxl1 was found to be localized to several areas within the seminiferous tubules, including immature spermatozoa tails, the cytoplasm of Sertoli cells, the basement membrane of the seminiferous tubule, blood vessels, and acrosomes. The most intense staining was localized to the acrosomes. Interestingly, the localization of Loxl1 to the acrosome is similar to the localization of clusterin, a glycoprotein that is produced constitutively by Sertoli cells and has been well-studied in the testis [21]. Clusterin has been found to localize to the cytoplasm of Sertoli cells, in the heads and tails of late spermatids and released spermatozoa, and at the acrosome [21, 22]. Recently, Loxl1 and clusterin have been identified as major genetic variants in pseudoexfoliation syndrome, which have been shown to co-localize in ocular tissues of patients with this disease [23, 24]. There is a possibility that these proteins co-localize in the testis as well, given the similar areas of localization. Further research will need to be performed to confirm this and also to determine the potential relationship of these proteins in the testis. The potential interactions of Loxl1 and clusterin in the testis at the protein level may also extend to the functional level, as clusterin has been implicated in protecting surviving cells after damage.

In summary, these studies have revealed candidate genes underlying the synergistic disruption of spermatogenesis that occurs following HD and CBZ co-exposure. CBZ and HD interact in an agonistic way at the gene level, reflective of the agonistic effects on testicular toxicity. Loxl1 and Clca2/Clca4l are both reduced in expression following co-exposure and appear to play critical roles in the testis. Further investigation is needed to determine the specific roles of Loxl1 and Clca2/Clca4l in the testicular response to damage. The results of the current study have also demonstrated, for the first time, the localization of Loxl1 in the testis. Additional mechanistic studies will reveal the functional significance of this localization in the testis as well as the testicular role of Loxl1, both under normal conditions and following toxicant exposure.

Research Highlights

- CBZ and HD interact in a synergistic way at both the histological level and at the gene level

- Combined exposure to HD and CBZ has synergistic effects on the expression of specific genes, including *Clca2/Clca4l* and *Lox11*.
- The localization of *Lox11* within the testis has not been previously investigated and *Lox11* was found to be localized to several areas within the seminiferous tubules with the most intense staining localized to the acrosomes.

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Abbreviations

HD	2,5-hexanedione
x-ray	x-radiation
CBZ	carbendazim
ms	milliseconds
h	hours
FDR	false discovery rate
ANOVA	one-way analysis of variance

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Figure 1.

Co-exposure effects on gene expression. Relative gene expression following exposure to 0% (blue line), 0.33% (green line) or 1% HD (red line) in combination with 0, 67 or 200 mg/kg CBZ was determined by microarray analysis using tissues obtained at 3 h following CBZ treatment.

Figure 2.

Gene expression 3 h after HD and CBZ co-exposure. mRNA was isolated from testis tissue after 1% HD, 200 mg/kg CBZ, or 1% HD + 200 mg/kg CBZ exposure and MacroD1, Dpp7, Shank3, Clca2/Clca4l, Loxl1, and Tubb3 expression was measured by qRT-PCR. Gene expression values are expressed as mean relative expression $\pm$ SEM ($n = 3-4$). Values above the results for each gene indicate the p value for the within-gene ANOVAs that were performed. * $p < 0.05$ compared to control, † $p < 0.05$ compared to HD, ‡ $p < 0.05$ compared to CBZ.

Figure 3.

Gene expression 24 h after HD and CBZ co-exposure. mRNA was isolated from testis tissue after 200 mg/kg CBZ or 1% HD + 200 mg/kg CBZ exposure and Shank3, Clca2/Clca4l, and Lox11 expression was measured by qRT-PCR. Gene expression values are expressed as mean relative expression $\pm$ SEM ($n = 3-4$). Values above the results for each gene indicate the p value for the within-gene ANOVAs that were performed.

Figure 4. Localization of Loxl1 protein 24 h following toxicant exposure. Rat testis sections were cut on a cryostat at 8 μ m thickness and prepared for Loxl1 protein immunofluorescent detection. Representative images of seminiferous tubules for each toxicant exposure: (A) Control (B) 1% HD (C) 200 mg/kg CBZ (D) 1% HD + 200mg/kg CBZ. The scale bar is 50 μ m, A–D.

Figure 5. Quantification of Lox11 Staining. Lox11 protein levels were quantified in immunofluorescent-stained tissue sections 24 h following control or toxicant (1% HD, 200mg/kg CBZ or 1% HD + 200mg/kg CBZ) exposure, n=6. Protein levels were measured as mean gray value, representing the mean gray value of the acrosome divided by the mean gray value of the seminiferous tubule basement membrane. ANOVA p=0.35

Table 1Genes with significant HD effects ($q < 0.05$)

Gene Symbol	Gene Description	HD Effect	Linear CBZ Effect	Type of Interaction
DPP7	dipeptidylpeptidase 7	-1.9670	-0.1638	Agonistic
MACROD1	MACRO domain containing 1	-2.2351	-0.2211	Agonistic
SHANK3	SH3 and multiple ankyrin repeat domains 3	-2.3335	-0.0784	Agonistic
CLCA2 /// CLCA4L	chloride channel calcium activated 2 /// chloride channel calcium activated 4-like	-3.1066	-0.7292	Agonistic
LOXL1	lysyl oxidase-like 1	-3.2776	-0.2296	Agonistic

Table 2Genes with significant CBZ effects ($q < 0.05$)

Gene Symbol	Gene Description	Linear CBZ Effect	HD Effect
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	0.7170	1.8503
GPI	glucose phosphate isomerase	-0.2068	-0.7908
AFG3L1	AFG3(ATPase family gene 3)-like 1 (<i>S. cerevisiae</i>)	-0.2283	-0.0024
ADIPOR2	adiponectin receptor 2	-0.3353	-0.4796
RECQL5	RecQ protein-like 5	-0.3957	-0.4150
RGD1564036	similar to RIKEN cDNA 3010026O09	-0.4380	1.2676
FALZ	fetal Alzheimer antigen	-0.5206	-0.9260
DCXR	dicarbonyl L-xylulose reductase	-0.5393	-0.6166
XTP3TPA	XTP3-transactivated protein A	-0.5552	-0.8730
CD164L2	CD164 sialomucin-like 2	-0.5785	-0.7292
FBXO46	F-box protein 46	-0.5815	0.2491
RGD1307155	similar to CG18661-PA	-0.6127	-0.9075
CLCA2 /// CLCA4L	chloride channel calcium activated 2 /// chloride channel calcium activated 4-like	-0.7292	-3.1066
FBXL15	F-box and leucine-rich repeat protein 15	-0.7509	0.3523
MMP9	matrix metalloproteinase 9	-0.7558	-1.3143
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	-1.0051	0.4993